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PREPARATION AND CHARACTERISATION OF PERMETHYLATED DERIVATIVES OF BILE ACIDS, AND THEIR APPLICATION TO GAS CHROMATOGRAPHIC ANALYSIS*

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SUMMARY

Permethylation of bile acids was achieved in a one-step reaction with sodium methylsulfinylmethanide and methyl iodide in dimethylsulfoxide. The products were characterised by proton nuclear magnetic resonance spectroscopy and by gas—liquid chromatography—mass spectrometry. The derivatives were separated by gas—liquid chromatography on a SP-2250 wall coated 30-m glass capillary column: formation of permethylated products was quantitative over the range 5—50 nmol for each bile acid.

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INTRODUCTION

Analysis of unconjugated bile acids by gas–liquid chromatography (GLC) requires derivatisation of both carboxyl and hydroxyl groups. Previously this has been accomplished in two stages. Methyl esters are first formed in methanolic hydrochloric acid [1], or by the action of diazomethane [2]. After removal of these reagents, derivatisation of the hydroxyl groups is then carried out to form acetates [3] or trimethylsilyl ethers [4]. These methods can result in handling losses, e.g. extraction and transfer of each sample, and in the case of the trimethylsilyl ethers a susceptibility to hydrolysis.

In this study we report the formation of highly stable bile acid methyl ester methyl ether derivatives. These were prepared in a one-step reaction using the Hakomori reaction [5], a method that has been extensively used in the structural analysis of polysaccharides [5, 6].

MATERIALS AND METHODS

Bile acids were obtained from Research Plus Labs. (Denville, NJ, U.S.A.). Dimethylsulfoxide was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Silicic acid was obtained from Mallinckrodt (St. Louis, MO, U.S.A.) and sodium hydride from Ventron (Danvers, MA, U.S.A.). All other reagents were purchased from Fisher (Norcross, GA, U.S.A.).

Synthesis of permethylated bile acids

Bile acids were purified by silicic acid column chromatography using mixtures of hexane and ethyl acetate as the eluting solvent. The following reaction procedure was used for each bile acid. A specimen (up to 50 mg) of each was carefully dried in a 3-ml reacti-vial (Reliance Glassworks, Bensenville, IL, U.S.A.) in vacuo over phosphorus pentoxide. A triangular magnetic stirrer was placed in each vial which was then sealed with a septum cap closure. Using two 25-gauge needles, the air in each vial was flushed out with nitrogen. The nitrogen was an oxygen-free grade (Hamilton Oxygen, Birmingham, AL, U.S.A.) and was purified by passage through a molecular sieve filter. Anhydrous dimethylsulfoxide, distilled over calcium hydride, was injected through the septum, while maintaining the nitrogen flow. Sodium methylsulfinylmethanide, prepared by reaction between anhydrous dimethylsulfoxide and sodium hydride [5], was added to the vial, and mixing continued for 1 h. This formed the alkoxides of all the hydroxyl groups. Methyl iodide, purified by shaking with mercury, was added in small aliquots, 10% of that required to neutralise the sodium methylsulfinylmethanide, was added each time. Nitrogen flow was stopped during this period to prevent loss of methyl iodide prior to reaction with the reagent. Excess methyl iodide was added to consume all the reagent and mixing continued until the mixture was neutral. In some experiments the excess methyl iodide was blown off with nitrogen. Methylation was repeated with addition of more sodium methylsulfinylmethanide, and subsequently, more methyl iodide. The permethylated products were extracted by the addition of 1 ml hexane and vigorous shaking. The upper hexane phase was

removed and the extraction repeated. The combined hexane phases were evaporated to dryness under nitrogen.

Purification of the permethylated products was carried out by preparative silicic acid column (25 × 1 cm) chromatography. Mixtures of hexane—ethyl acetate were used as eluting solvent, with the ethyl acetate proportion varying between 5 and 20%. Fractions containing the permethylated material were combined and evaporated to dryness. The derivatives were recrystallised from hexane.

In order to recover all the products, including unreacted material, the reaction mixture was acidified with 4 *M* hydrochloric acid and extracted with 2 ml ethyl acetate twice. Iodine, which was also extracted was removed by sublimation after evaporation of the ethyl acetate. Uncorrected melting point determinations were performed using a Fisher—Johns melting point apparatus.

Evidence for permethylation

The term permethylated refers to a derivative in which all available hydroxyl groups (to form methyl ethers) and the carboxylic acid group (to form a methyl ester) have been methylated.

To measure the extent of methylation, two techniques were used.

For chenodeoxycholic acid ($3\alpha,7\alpha$-dihydroxy-5β-cholan-24-oic acid) 1 μCi of the 11,12-^{3}H-labelled material (Amersham-Searle, Arlington Heights, IL, U.S.A.) was added to the reaction. The permethylated material was analysed by thin-layer chromatography (TLC) on 0.25 mm thick silica gel G 20 × 20 cm plates (Rediplates, Fisher) using hexane—ethyl acetate (4:1, v/v) at 25°C. Duplicate tracks were sprayed with Usui's reagent [7], 3% (w/v) phosphomolybdic acid in glacial acetic acid—sulfuric acid (19:1, v/v), and heated at 100°C for 5 min to locate the bile acids. Each track was divided into 1-cm zones and the silica scraped into plastic scintillation vials. Radioactivity was measured following the addition of 0.5 ml methanol and 5 ml NE-260 scintillant (Nuclear Enterprises, San Carlos, CA, U.S.A.) using a Packard 2450 liquid scintillation counter (Downers Grove, IL, U.S.A.).

In the case of the other bile acids, the extent of methylation was determined by treatment of a portion of the ethyl acetate extract with pyridine—hexamethyldisilazane—trimethylchlorosilane (4:2:1, v,v) at 37°C for 30 min. The resulting derivatives were extracted into hexane after addition of 1 ml hexane and 0.5 ml of water. The hexane layer was evaporated to dryness after centrifugation and dissolved in toluene for analysis by GLC.

Gas—liquid chromatography

GLC analysis was performed using a 30 m × 0.2 mm wall-coated SP-2250 glass capillary (Scientific Glass Engineering, Houston, TX, U.S.A.) mounted in a Hewlett-Packard (Palo Alto, CA, U.S.A.) 5831 gas chromatograph. The conditions for analysis were as follows; helium carrier gas, 2 ml/min; inlet split ratio 10:1; make-up nitrogen gas, 30 ml/min, hydrogen, 40 ml/min and air 300 ml/min; temperatures: inlet, 300°C; column, 250°C and flame ionisation detector, 300°C. Additional studies were carried out using a splitless injector. The column was a 30 m × 0.2 mm wall-coated SP-2100 glass capillary. The temperature program used was 80°C for 0.5 min, 80—270°C at 20°C/min, and finally,

isothermal at 270°C. Acetate ester methyl ester derivatives of bile acids were prepared by the method of Roovers et al. [3].

Quantitative studies were carried out using mixtures of bile acids, with the mass of each varying from 5—50 nmol. The internal standard, 5 nmol 5α-cholestane, was added to the reaction mixture which was extracted with hexane. The hexane was evaporated to dryness and redissolved in 50 μl toluene. Aliquots (1 μl, 100—1000 pmol) were analysed on the SP-2250 capillary column. Linear regression analysis was used to relate the area response (relative to 5α-cholestane) and the mass of each bile acid being derivatised.

Mass spectrometry

Gas—liquid chromatography—mass spectrometry (GLC—MS) was performed on a Hewlett-Packard 5985 GC—MS instrument, with a 15 m × 0.2 mm SP-2250 wall-coated glass capillary. Splitless injection was used to introduce the samples and the temperature program 80°C for 0.5 min, 80—270°C at 20°C/min, and finally, isothermal at 270°C. The source temperature was maintained at 200°C. The ionising electron energy was 70 eV. Spectral data were acquired by an attached data system, and were processed to remove contributions from column bleed.

Nuclear magnetic resonance spectroscopy

Proton spectra were measured at 400 MHz using a Bruker WH-400 nuclear magnetic resonance spectrometer. Crystalline permethylated bile acids were kept in vacuo to remove solvents; then 5 mg (0.01 mmol) was dissolved in 1 ml deuterochloroform for measurement, tetramethylsilane being added as internal reference.

RESULTS

Methylation of each of the bile acids led to single products as judged by GLC and by TLC. An oily residue, probably arising from the mineral oil associated with the sodium hydride, was removed by passage over small silicic acid columns which were eluted with hexane—ethyl acetate (1:1, v/v). Preparative silicic acid chromatography for three of the bile acid derivatives yielded material which crystallised readily from hexane. Melting points were determined as follows: lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) 81—83°C, 3β-hydroxychol-5-enoic acid, 106—108°C and chenodeoxycholic acid, 127—128°C. The derivative of cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) only crystallised when kept in vacuo for one week. The crystalline product melted at 60°C, suggesting that it was highly solvated.

Nuclear magnetic resonance spectroscopy

Proton spectra of the crystalline derivatives at 400 MHz were determined in deuterochloroform. The methyl ether groups gave prominent resonances. The 3α-OCH_3 in the lithocholic acid derivative and the 3β-OCH_3 in 3β-hydroxychol-5-enoic acid derivative were 3.35 ppm downfield from the internal standard, tetramethylsilane. For the chenodeoxycholic acid derivative, the 3α-OCH_3 group was at 3.33 ppm and the 7α-OCH_3 group at 3.23 ppm. In the case of the

cholic acid derivative, the 3α-OCH_3 was at 3.33 ppm, the 7α-OCH_3 at 3.25 ppm and the 12α-OCH_3 at 3.19 ppm (Fig. 1). The methyl ester proton resonance was at 3.65 ppm.

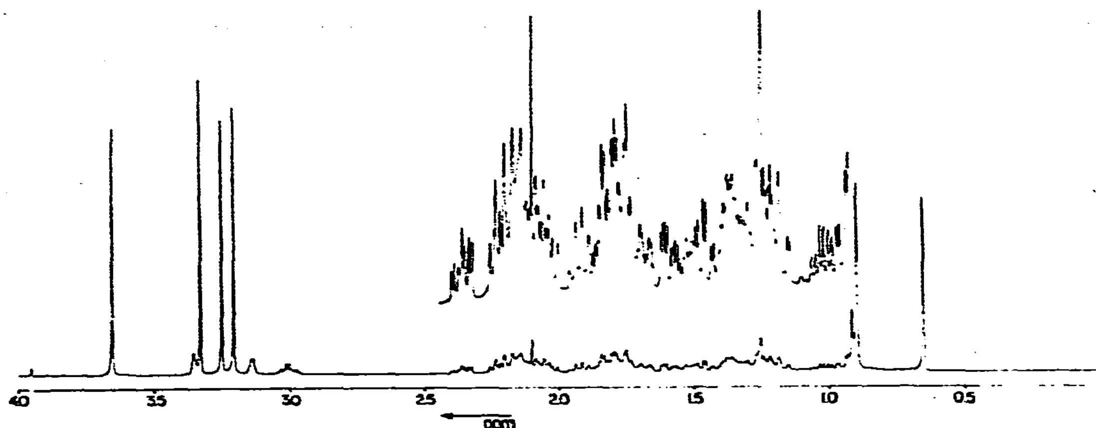

Fig. 1. Proton 400 MHz nuclear magnetic resonance spectrum of permethylated cholic acid. The methyl ether resonances are those at 3.33 ppm, 3.25 ppm and 3.19 ppm, the 3α-, 7α- and 12α-positions respectively. The resonance at 3.65 ppm is from the methyl ester protons. The large number of resonances between 1.0 and 2.5 ppm are from protons in the steroid nucleus.

Mass spectrometry

Electron ionisation mass spectra were obtained following GLC. These included the four crystalline materials and three other bile acids which were not purified prior to GLC. Fragments previously characterised for other bile acid derivatives [8, 9] were observed which represented loss of functional groups (*m/z* 368, 370, 372) and the additional loss of the side chain, C_{20-24} (*m/z* 253, 255, 257). For permethylated bile acids, fragments 32 mass units larger, representing retention of a methyl ether group, were also found. Permethylated derivatives of lithocholic acid (*m/z* 404), 3β-hydroxychol-5-enoic acid (*m/z* 402), and ursodeoxycholic (3α,7β-dihydroxy-5β-cholan-24-oic acid) and hyodeoxycholic acids (3α,6α-dihydroxy-5β-cholan-24-oic acid) (*m/z* both at 434) gave molecular ions (Fig. 2).

Quantitative aspects

The completeness of methylation was investigated using [11,12-^{3}H]chenodeoxycholic acid and 3 mg unlabelled chenodeoxycholic acid. When the necessary amount of methyl iodide was added in one addition, methylation was only 50—80% complete as evidenced by local heating and evolution of gas. In contrast, addition of methyl iodide in small amounts gave a yield in excess of 99.5%, as assessed by TLC analysis. This result was confirmed by treating the hexane fraction to form trimethylsilyl ethers of any unreacted hydroxyl groups. A small amount of 3α-methoxy-7α-trimethylsilyl ether of methyl chenodeoxycholate (0.2%) was found. As noted by others [6], there is no substitute for scrupulous attention to maintaining dry and oxygen-free conditions in order to obtain complete methylation.

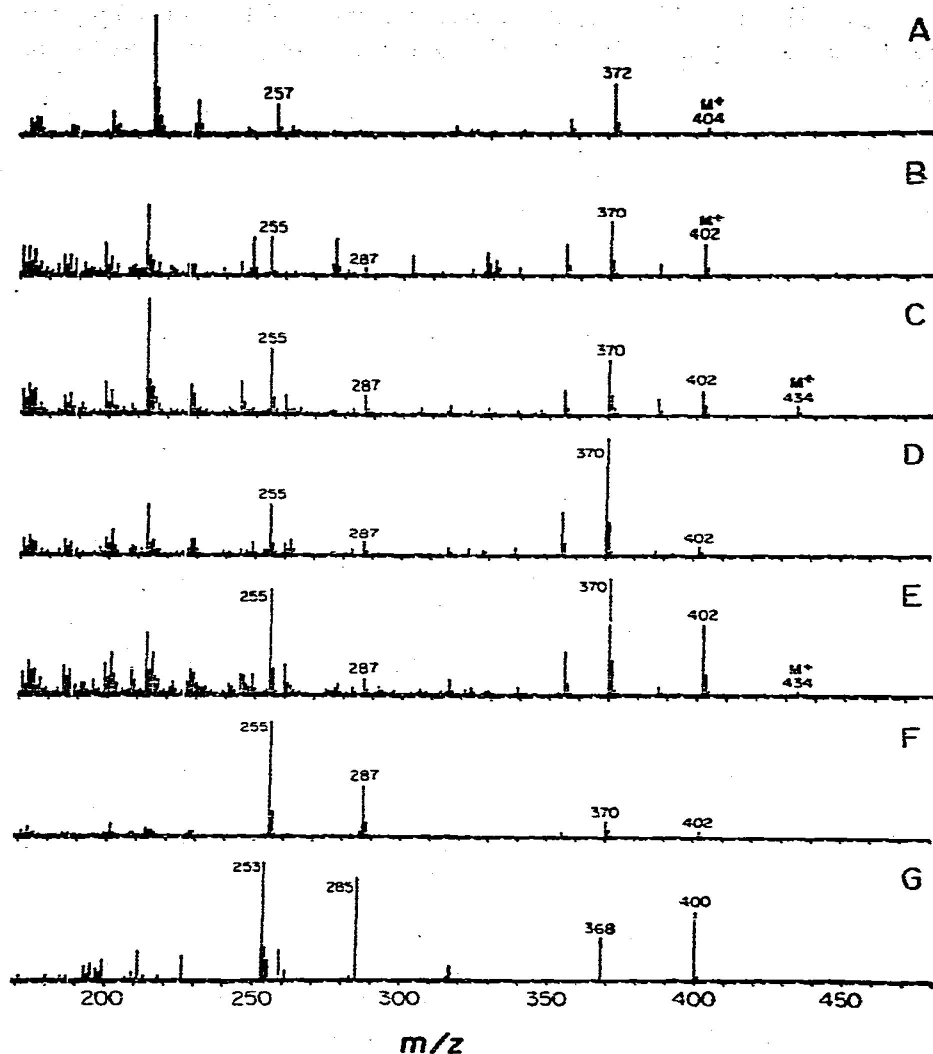

Fig. 2. Electron ionisation mass spectra of seven permethylated bile acids. Identification: A = lithocholic acid; B = 3β-hydroxychol-5-enoic acid; C = hyodeoxycholic acid; D = chenodeoxycholic acid; E = ursodeoxycholic acid; F = deoxycholic acid (3α-12α-dihydroxy-5β-cholan-24-oic acid) and G = cholic acid.

The applicability of the method was investigated in the range 5—50 nmol, using a mixture of seven bile acids. The response curve (by area relative to that of the internal standard 5α-cholestane) was linear for each bile acid as shown for chenodeoxycholic acid in Fig. 3. The relative molar responses (in arbitary units) were close and ranged from 0.113 to 0.120.

Using splitless injection, 50 pmol (20 ng) of each bile acid could be detected with a stable baseline (Fig. 4). For comparison, acetate ester methyl ester derivatives of each bile acid were added to the sample (Fig. 4).

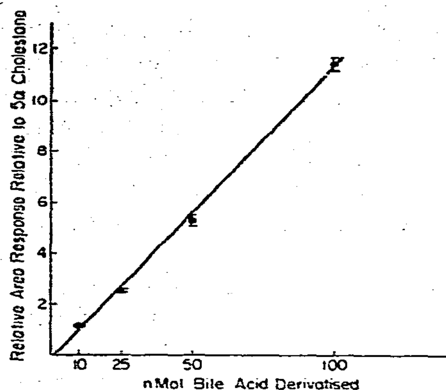

Fig. 3. Response curve of permethylated chenodeoxycholic acid. Response was determined on basis of peak area relative to the internal standard, 5α-cholestane. Data shown are mean ± S.D. (n = 4).

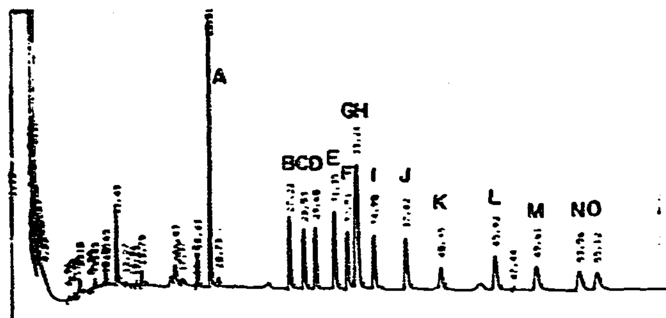

Fig. 4. Capillary gas—liquid chromatogram of bile acid derivatives. Each of the bile acids (50 pmol) was analysed by splitless injection on a wall-coated SP-2100 30 m × 0.2 mm glass capillary column. The temperature program 80°C for 0.5 min, 80—270°C at 20°C/min, and finally, isothermal at 270°C was used. Also included in the sample were the corresponding acetate ester methyl ester derivatives. Peaks: A = 5α-cholestane; B,I = lithocholic acid; C,J = 3β-hydroxychol-5-enoic acid; D,K = deoxycholic acid; E,L = chenodeoxycholic acid; F,O = ursodeoxycholic acid; G,N = hyodeoxycholic acid; H,M = cholic acid. The first letter of each pair corresponds to the permethylated derivative, the second letter to the acetate ester methyl ester derivative.

DISCUSSION

Using the Hakomori reagent [5], sodium methylsulfinylmethanide, and methyl iodide, bile acids are readily converted in a one-step reaction to permethylated derivatives. This type of derivative has been prepared previously [10—13], but by inferior methods. Partially methylated derivatives of chenodeoxycholic acid were produced as unwanted by-products during methyl ester formation using ethereal diazomethane [10]. The permethylated form of 3β-7α-dihydroxychol-5-enoic acid has been prepared using methanol—hydrochloric acid in order to overcome the problems of measuring this bile acid [11, 12]. The permethylated derivative of lithocholic acid has been made recently using potassium *tert.*-butoxide and methyl iodide [13].

The permethylated derivatives are very stable and can be readily separated by capillary GLC. Electron impact ionisation mass spectra gave rise to fragments specific for the presence of the methyl ether group. Similar fragments have been found for the 3α-methoxy-7α,12α-diacetoxy derivative of cholic acid methyl ester [8]. Permethylated derivatives of several bile acids gave rise to readily discernible mass ions (M^+), indicating the increased stability of the molecular ion of this type of derivative over the acetate esters and trimethylsilyl ether derivatives.

The formation of the permethylated derivatives was quantitative and reproducible on the micro scale, the response curve being linear over the range 5—50 nmol. Other studies, not presented here, have extended the linearity into the pmol range. Using splitless injection techniques, amounts as little as 1—5 pmol (injected) can be satisfactorily separated and quantified. Thus without resort to electron-capture detection, sensitivity which approximates that of the bile acid radioimmunoassays [14, 15] can be achieved.

The high degree of stability of permethylated derivatives and the advantages of their single-step formation makes these derivatives excellent candidates for quantitative analysis. In an analogous manner to structural analysis of carbohydrates, methyl ether methyl ester derivatives can be used to determine the position and orientation of sulfate groups in the bile acid molecule [16].

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